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(54) Title: ANTI-HIV ACTIVITY OF LIPOSOMAL NYSTATIN AND AMPHOTERICIN B

(57) Abstract

A method is disclosed for inhibiting the replication of HIV-1 virus. The method includes the step of administering to a subject which is infected with HIV-1 a pharmaceutically effective amount of a liposomal composition which comprises at least one lipid and a polyene selected from nystatin and amphotericin B. Such liposomal compositions have been found to be selectively toxic for HIV-infected cells. The composition preferably comprises nystatin, dimyristoyl phosphatidyl choline, and dimyristoyl phosphatidyl glycerol, with the weight ratio of the latter two components being about 7:3.

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ANTI-HIV ACTIVITY OF LIPOSOMAL NYSTATIN AND AMPHOTERICIN B

The United States may own certain rights in this invention as the development of part of the present invention was supported by grant number NIH-NO1-AI-72639 from the National Institutes of Health, Department of Health and Human Services.

This application is a continuation in part of U.S. application serial number 314,710, filed on February 23, 1989. That application is incorporated here by reference.

Acquired immunodeficiency syndrome (AIDS), resulting from infection by the human immunodeficiency virus (HIV), affects thousands of people around the world, and is uniformly fatal. Extensive research efforts are underway in an attempt to develop methods of curing or preventing this disease, but as of the present, no completely satisfactory solution has been found.

One group of drugs with known antibiotic properties is the polyene macrolides, which are secondary metabolites produced by various species of Streptomyces. Without being bound by any particular theory of activity, the pr viously-known antibiotic properties of the polyenes appears to be based on their interaction with

sterols in the cell membranes of infectious agents, such as fungal cell membranes. As a result of this interaction, the membranes are rendered selectively permeable to the outflow of vital constituents, such as potassium.

Nystatin and amphotericin B are two specific examples of polyenes. Although they have previously demonstrated some antibiotic activity, they have not been previously demonstrated to have a practical use with respect to treatment or prophylaxis of AIDS. The present invention is based on the surprising discovery that certain formulations comprising nystatin or amphotericn B do in fact have practical and useful activity against HIV-1 virus.

The present invention, in one aspect, concerns a method for inhibiting the replication of HIV-1 virus, comprising the step of administering to a subject which is infected with HIV-1 a pharmaceutically effective amount of a liposomal composition which comprises a polyene selected from nystatin and amphotericin B. The composition will comprise liposomal vesicles, formed of at least one phospholipid, in which a polyene selected from nystatin and amphotericin B is entrapped or encapsulated. The composition can optionally also comprise a sterol, such as cholesterol.

In another aspect, the present invention relates to a pharmaceutical composition comprising a cytotoxicity-exclusionary amount of a liposomal polyene selected from nystatin and amphotericin B. In this respect, the invention is based on the therapeutic window created by the sel ctive activity of liposomal nystatin and amphotericin B, wh reby a dose of such compositions that is toxic to HIV-infected cells is not substantially toxic

to uninfected cells. "Cytotoxicity-exclusionary" means a composition or dosage which has an LD_{50} for HIV-infected target cells above the ID_{50} for noninfected target cells.

In another aspect, the present invention concerns a method of inhibiting the replication of HIV-1 virus, comprising the step of administering to a subject which is infected with HIV-1 a vector-interdicting amount of a composition which comprises liposomal vesicles in which a polyene selected from nystatin and amphotericin B is incorporated. A "vector-interdicting amount" refers to the amount which will result in the inhibition, blocking, or elimination of a vector pathway for HIV proliferation and/or transmission in the treatment of an animal. "Vector pathway" refers to organ and organelle (i.e., nonfluid) replication-based pathways of HIV proliferation and transmission.

In preferred embodiments of the invention, the lipids will make up between about 25% and about 90% by weight of the total amount of lipid and nystatin. If a sterol is included, it will preferably make up between about 10% and about 75% by weight, most preferably between about 30% and about 60%, of the total amount of nystatin, lipid, and sterol. In an especially preferred embodiment, the composition consists essentially of nystatin, dimyristoyl phosphatidyl choline, and dimyristoyl phosphatidyl glycerol, with the weight ratio of DMPC:DMPG being about 7:3. The weight ratio of nystatin to lipids is preferably about 1:10.

The present invention permits the selectively toxic treatment of an animal that is infected with HIV-1 virus, whereby infected cells are selectively killed, while avoiding substantial toxicity to other cells. The present invention takes advantage of the surprising

ability of liposomal nystatin and amphotericin B to inhibit the replication and/or transmission of HIV-1. This activity is especially surprising in view of the lack of such activity on the part of liposomal formulations of other polyenes, such as mepartricin and hamycin. The compositions and methods of the present invention should be useful for the treatment and/or prophylaxis of AIDS in animals, including humans.

In the method of the present invention, the agent to be administered comprises a polyene selected from the group consisting of nystatin and amphotericin B, at least one phospholipid, and optionally a sterol, such as cholesterol. The phospholipid takes the form of lipid vesicles or liposomes, in which the polyene is entrapped or encapsulated. (For the sake of simplicity, in this patent all lipid vesicles in which polyene is entrapped or encapsulated will be referred to as "liposomes".) The liposomes can be unilamellar, multilamellar, or can have an undefined lamellar construction.

The phospholipids are preferably selected from the group consisting of phosphatidyl choline, phosphatidyl serine, phosphatidyl glycerol, sphingomyelin, and phosphatidic acid. The most preferred phospholipids are dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol. However, other lipids can be used.

When the liposomes comprise dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol, those two lipids are preferably present in a weight ratio of between 1:10 and 10:1, with the most preferred ratio being 7:3 DMPC:DMPG. The lipids preferably comprise from about 25% to about 90 % by weight of the overall composition of lipid, nystatin, and optionally, sterol.

It is useful to include sterol in the composition in order to reduce the toxicity that is typically associated with free nystatin. The sterol, for example cholesterol, can be included in an amount equal to from 10% to 75% by weight of the overall composition (on a dry basis). A more preferred range is 30% to 60%.

The mode of administration is preferably parenteral, i.e., by intravenous, intraarterial, intramuscular, intralymphatic, intraperitoneal, subcutaneous, intrapleural, or intrathecal injection or infusion. The administration could also be through inhalation or topical or oral means. The composition can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable carrier substances which do not deleteriously react with the active compositions. The composition is preferably formulated as a pharmaceutically acceptable solution, such as a sterile isotonic aqueous solution. A pharmaceutically effective amount will be administered, preferably in a dosage between about 0.1 mg of nystatin/kg of body weight and about 80 mg/kg. The presently preferred dosage is about 4 mg/kg, administered three times a week for about six months.

Further details regarding the making and use of the present invention can be determined from the following examples.

Examples

Drugs. Nystatin (Nys) was obtained from American Cyanamid Company. Liposomal nystatin (L-Nys) was prepared as previously described. Briefly, the drug in methanol and the phospholipids, dimyristoyl phosphatidyl cholin (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) dissolved in chloroform (DMPC:DMPG, 7:3 by weight)

were mixed in a ratio of 1:10 (nys:phospholipid) and the organic solvents were evaporated. The dried lipid film was dispersed with 0.9% NaCl solution.

Another procedure for preparing the composition is as follows. 7 g of DMPC (Lipoid KG, Ludwigshafen, West Germany) and 3 g of DMPG (Lipoid KG) are mixed with 250 cc of t-butyl alcohol (Fisher Scientific, Fairlawn, New Jersey). 250 cc of water are added and mixed until dissolved. This solution is then passed through a 0.45 μM pore size filter cartridge. Separately, 1.1 g Nys is dissolved in 7 cc of methyl sulfoxide (Fisher Scientific), and mixed until dissolved. The nys solution is then passed through a 0.45 μM pore size filter cartridge, and the lipid solution and nys solution are then mixed. The concentration is adjusted to 1.1 mg/ml by adding an appropriate volume of diluent, and the solution is then passed through a sterile, 0.22 μM membrane. The solvents can be removed by lyophilization, and the resulting preliposomal powder can be reconstituted when desired by adding water or saline solution.

Virus. HIV-1 (HTLV-III/B) was prepared from HIV infected H9 cells in culture. Cells were harvested and the virus isolated from the culture medium by banding on sucrose gradients. The virus band sediment had a density of 1.16 to 1.19 g/ml, and was collected by pelleting. The pellet was resuspended in TNE (10mM Tris. HCl, pH 7.5, 0.1M NaCl, 0.001 EDTA) at $2x10^{11}$ virus particles/ml.

H9 Cells. H9 cells are a transformed line of human lymphocytes permissive for HIV-1. The cultures were maintained using RPMI supplemented with 20% fetal bovine serum, 2% glutamine and 1% gentamicin. Cells were

harvested in log phase of growth for testing and seeded at $5x10^5$ cells/test culture.

Culture Conditions. HIV-1 at $5x10^8$ particles/ml was added to the test cultures ($5x10^5$ cells/ml) simultaneously with compounds to be tested. The cultures were incubated for 4 days at 37°C in an atmosphere containing 5% CO_2. Cells were then harvested and assayed for virus growth as described below.

Immunofluorescence Assays. The cells were collected by centrifugation, washed and resuspended in 0.01M phosphate buffered saline (PBS), to approximately $1x10^6$ cells/ml. An aliquot was used for determining cell viability by the exclusion of trypan blue dye. The remaining cells were added to wells on a toxoplasmosis slide, allowed to dry for one hour and fixed in acetone methanol (1:1, v/v) for 15 minutes, as described. After fixation, the slides were pretreated with 10% normal goat serum at room temperature for 30 minutes. The slides were rinsed four times with PBS, 15 minutes each wash. Mouse monoclonal antibody to HIV-1 p24 antigen was added to each slide and incubated for 30 minutes at 37°C, in a humidified chamber. The cells were then rinsed with PBS, and washed 4 times as above. The cells were then stained with FITC labeled goat anti-mouse IgG by incubating for 30 minutes as above.

The slides were rinsed with PBS, washed 4 times and given a final wash overnight with fresh PBS. The slides were counterstained with 0.02% Evans blue for 1 minute followed by rinsing with distilled water. After air-drying the slides were mounted with 50% glyc rol, and fluorescence m asurements were done with a Zeiss fluorescenc microscope. The results are expressed as

the p rcent of fluorescent positive cells derived from the average of several microscopic fields.

Reverse Transcriptase Activity. The supernatant fractions of the test cultures were assayed for reverse transcriptase activity as described previously (Sarin, 1985, 1986). This was measured by the incorporation of ^{3}H-deoxythymidine triphosphate into trichloracetic acid-insoluble DNA using $(dT)_{15}:(A)_n$, as a template primer.

Antibody Induction Assay. C57BL/6 mice were primed with $1x10^7$ sheep red blood cells (SRBC's) ip 10 days prior to the experiment. The mice were sacrificed, spleens removed and a homogeneous cell population prepared as using a tissue homogenizer. Cultures were set up with $1x10^7$ primed spleen cells, $1x10^5$ SRBC's and 0.1 ml of the test compound. The cultures were incubated for 6 days in Click's medium (Click, 1972) under the same conditions as the H9 cultures. After incubation, the supernatant fluids were collected by centrifugation and assayed for the presence of antibody to sheep red blood cells by the complement dependent immune hemolysis assay.

Complement Dependent Immune Hemolysis. Various dilutions (1:10, 1:100, 1:1000) of the culture supernatants were added to $1x10^8$ SRBC's for 30 minutes at room temperature. The cells were collected by centrifugation, washed with vernal buffer, and mixed with an excess of complement (Gibco) determined by hemolysin titration. The suspensions were incubated for 1 hour in a 37°C water bath. After incubation the supernatants were collected by centrifugation and read spectrophotometrically at 541 nm for the presence of hemoglobin from the lysed SRBC's. This direct test measures the amount of IgM in th cultures. To measure IgG, total antibody was m asured by an indirect technique using rabbit anti-mouse IgG. The

antibody was added to the suspensions before the addition of complement to facilitate complement binding. The value obtained from the indirect technique is subtracted from the total antibody and the result is the amount of IgG in the culture. The unit of antibody is defined as the amount of antibody which can sensitize 50% of the SRBC's lysed in the presence of complement.

Table 1. Anti-HIV-1(IIIB) activity of liposomal-nystatin

Drug	Drug Combination	H9 cells	Percent inhibition of HIV expression		
	µg/ml	(10^6/ml)	p17	p24	RT
H9 cells (no virus)		2.52			
H9 cells infected		1.72			
Experiment 1					
Empty liposomes	0.01		0	0	0
	0.1		0	0	0
	1.0		0	0	0
	10.0		0	0	0
	100.0		0	0	0
Liposomal-Nystatin	0.01	2.36	0	0	26
	0.1	2.72	35	33	27
	1.0	1.76	50	40	30
	10.0	1.6	90	93	70
	100.0	Toxic	Toxic to cells		
Experiment 2					
Empty liposomes	0.01		0	0	0
	0.1		0	0	0
	1.0		0	0	0
	10.0		0	0	0
	100.0		0	0	0
Liposomal-Nystatin	0.01		0	0	0
	0.1		39	31	44
	1.0		54	37	35
	10.0		92	99	78
	100.0		Toxic to cells		

Table 2. Anti-HIV-1_{cc} activity of liposomal-nystatin

Drug	Drug Combination		Percent inhibition of HIV expression		
	μg/ml	Syncytia inhibition	p17	p24	RT
Experiment 1					
Empty liposomes	7.5	0	0	0	0
	10	0	0	0	0
	15	0	0	0	0
	20	0	0	0	0
Liposomal-Nystatin	7.5	0	29	57	52
	10	60	55	83	69
	15	88	81	90	72
	20	89	81	93	88
Experiment 2					
Empty liposomes	7.5	0	0	0	0
	10	0	0	0	0
	15	0	0	0	0
	20	0	0	0	0
Liposomal-Nystatin	7.5	18	39	60	52
	10	58	61	77	62
	15	82	74	83	80
	20	80	82	90	83

Anti-HIV (IIIB) activity of L-Nys in H9 cells

In the first set of experiments, in which the effects of L-Nys on HIV (IIIB) infected H9 cells was tested, the viral antigens p17 and p24 were inhibited in a dose dependent fashion by L-Nys (see Table 1). RT activity was also inhibited to a general degree. Empty liposomes had no effect on any of the parameters studied. L-Nys was not toxic to uninfected H9 cells at concentrations up to 10 μg/ml; however, at concentrations between 10.0-100.00, L-Nys was toxic to HIV infected cells. HIV-expression was inhibited by L-Nys at concentrations which are not toxic to uninfected H9 cells.

Anti-HIV-1_{cc} activity of L-Nys in Molt-3 cells

When the effects of L-Nys on HIV-1_{cc} infected Molt-3 cells was tested, similar to H9 cells, a dose response effect on p17, p24 and RT was observed (see Table 2). Furthermore, syncytia formation was also inhibited. Empty liposomes did not have an effect on the parameters studied. L-Nys was not toxic to non-infected Molt-3 cells at the concentrations studied.

The results obtained indicate that L-Nys displayed a selective toxicity for HIV infected cells. L-Nys had direct anti-HIV activity as reflected in the inhibition of syncitia formation, and on HIV infected cells. L-Nys was not toxic even at the higher concentrations used on non-infected cells. The HIV inhibition observed was dose dependent for all parameters tested.

The increased cholesterol observed in HIV-infected cells may make the infected cells more vulnerable to the membrane effects of Nys. We have previously shown that L-Nys was far less toxic to human red blood cells than fr e Nys, thus liposomal incorporation prevented the

direct toxic effects of Nys to mammalian cells. See U.S. patent 4,812,312, which is incorporated here by reference. Such was also the case for non-infected lymphocytes.

* * *

The preceding description is intended to illustrate specific embodiments of the present invention. It is not intended to be an exhaustive list of all possible embodiments. Person skilled in this field will recognize that modifications could be made to the compositions and methods described above that would remain within the scope of the present invention.

CLAIMS:

1. A method of inhibiting the replication of HIV-1 virus, comprising the step of administering to a subject which is infected with HIV-1 a pharmaceutically effective amount of a composition which comprises liposomal vesicles in which a polyene selected from nystatin and amphotericin B is incorporated.

2. The method of claim 1, where the composition further comprises a sterol.

3. The method of claim 2, where the sterol is cholesterol.

4. The method of claim 2, where the sterol makes up between about 10% and about 75% by weight of the total amount of polyene, phospholipid, and sterol in the composition.

5. The method of claim 2, where the sterol makes up between about 30% and about 60% by weight of the total amount of polyene, phospholipid, and sterol in the composition.

6. The method of claim 1, where the mode of administration is parenteral.

7. The method of claim 1, where the composition comprises at least one phospholipid selected from

phosphatidyl choline, phosphatidyl serine, phosphatidyl glycerol, sphingomyelin, and phosphatidic acid.

8. The method of claim 1, where the phospholipids in the composition consist essentially of dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol in a weight ratio of between about 1:10 and about 10:1.

9. The method of claim 8, where the ratio of DMPC to DMPG is about 7:3.

10. The method of claim 1, where the phospholipids make up between about 25% and about 90% by weight of the total amount of polyene and phospholipids.

11. A method of inhibiting the replication of HIV-1 virus, comprising the step of administering to a subject which is infected with HIV-1 a pharmaceutically effective amount of a composition which comprises liposomal vesicles in which nystatin is incorporated.

12. The method of claim 11, where the composition further comprises a sterol.

13. The method of claim 12, where the sterol is cholesterol.

14. The method of claim 12, where the sterol makes up between about 10% and about 75% by weight of the total amount of nystatin, phospholipid, and sterol in the composition.

15. The method of claim 12, where the sterol makes up between about 30% and about 60% by weight of the total amount of nystatin, phospholipid, and sterol in the composition.

16. The method of claim 11, where the mode of administration is parenteral.

17. The method of claim 11, where the composition comprises at least one phospholipid selected from phosphatidyl choline, phosphatidyl serine, phosphatidyl glycerol, sphingomyelin, and phosphatidic acid.

18. The method of claim 11, where the phospholipids in the composition consist essentially of dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol in a weight ratio of between about 1:10 and about 10:1.

19. The method of claim 18, where the ratio of DMPC to DMPG is about 7:3.

20. The method of claim 11, where the phospholipids make up betw en about 25% and about 90% by weight of the total amount of nystatin and phospholipids.

21. A method of inhibiting the replication of HIV-1 virus, comprising the step of administering to a subject which is infected with HIV-1 a pharmaceutically effective amount of a composition which comprises nystatin incorporated in liposomes, where the liposomes comprise phospholipids which consist essentially of dimyristoyl phosphatidyl glycerol and dimyristoyl phosphatidyl choline.

22. The method of claim 21, where the ratio of DMPC to DMPG is about 7:3 by weight.

23. The method of claim 21, where the composition further comprises cholesterol.

24. The method of claim 23, where cholesterol makes up between about 30% and about 60% of the total amount of nystatin, phospholipid, and cholesterol in the composition.

25. The method of claim 21, where the phospholipids make up between about 25% to about 90% by weight of the total amount of phospholipid and nystatin.

26. The method of claim 21, where the composition is administered to the subject in a dosage of about 4 mg nystatin per kg of body weight.

27. A method of inhibiting the replication of HIV-1 virus, comprising the step of parenterally administering to a subject which is infected with HIV-1 a pharmaceutically effective amount of a composition which comprises liposomal vesicles in which nystatin is incorporated, where the vesicles consist essentially of dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol in a weight ratio of about 7:3, and where the weight ratio of lipids to nystatin is about 10:1.

28. A cytotoxicity-exclusionary pharmaceutical composition comprising a cytotoxicity-exclusionary amount of a liposomal polyene selected from nystatin and amphotericin B.

29. The composition of claim 28 where the composition further comprises a sterol.

30. The composition of claim 29, where the sterol is cholesterol.

31. The composition of claim 29, where the sterol makes up between about 10% and about 75% by weight of the total amount of polyene, phospholipid, and sterol in the composition.

32. The composition of claim 29, where the sterol makes up between about 30% and about 60% by weight of the total amount of polyene, phospholipid, and sterol in the composition.

33. The composition of claim 28, where the composition comprises at least one phospholipid selected from phosphatidyl choline, phosphatidyl serine, phosphatidyl glycerol, sphingomyelin, and phosphatidic acid.

34. The composition of claim 33, where the phospholipids in the composition consist essentially of dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol in a weight ratio of between about 1:10 and about 10:1.

35. The composition of claim 34, where the ratio of DMPC to DMPG is about 7:3.

36. The composition of claim 28, where the phospholipids make up between about 25% and about 90% by weight of the total amount of polyene and phospholipids.

37. A method for treatment of an HIV-infected animal, comprising administering to the animal a cytotoxicity-exclusionary pharmaceutical composition comprising a liposomal polyene selected from nystatin and amphotericin B.

38. The method of claim 37, where the cytotoxicity-exclusionary composition is administered in an HIV vector-interdicting dosage.

39. A method of inhibiting the replication of HIV-1 virus, comprising the step of administering to a subject which is infected with HIV-1 a vector-interdicting amount of a composition which comprises liposomal vesicles in which a polyene selected from nystatin and amphotericin B is incorporated.

40. The method of claim 39, where the composition further comprises a sterol.

41. The method of claim 40, where the sterol is cholesterol.

42. The method of claim 40, where the sterol makes up between about 10% and about 75% by weight of the total amount of polyene, phospholipid, and sterol in the composition.

43. The method of claim 40, where the sterol makes up between about 30% and about 60% by weight of the total amount of polyene, phospholipid, and sterol in the composition.

44. The method of claim 39, where the mode of administration is parenteral.

45. The method of claim 39, where the composition comprises at least one phospholipid selected from phosphatidyl choline, phosphatidyl serine, phosphatidyl glycerol, sphingomyelin, and phosphatidic acid.

46. The method of claim 39, where the phospholipids in the composition consist essentially of dimyristoyl phosphatidyl choline and dimyristoyl phosphatidyl glycerol in a weight ratio of between about 1:10 and about 10:1.

47. The method of claim 46, where the ratio of DMPC to DMPG is about 7:3.

48. The method of claim 39, where the phospholipids make up between about 25% and about 90% by weight of the total amount of polyene and phospholipids.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/00057

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 9/127 A 61 K 31/71

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	Antiviral Research, vol. 11, no. 3, April 1989, Elsevier Science Publishers B.V. (Biomedical Division), D.R. PONTANI et al.: "Inhibition of HIV replication by liposomal encapsulated amphotericin B", pages 119-126, see the whole document ---	1-10, 3 -48
Y	---	11-27
Y	Biochemical Pharmacology, vol. 35, no. 22, 1986, Pergamon Journals Ltd, (GB), J.E. DAHLBERG et al.: "Anti-viral activity of amphotericin B methyl ester: inhibition of HTLV-III replication in cell culture", pages 4110-4113, see the whole document --- -/-	11-27

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
01-04-1992	28 APR 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme N. KUIPER

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication. where appropriate, of the relevant passages	Relevant to Claim No.
Y	W. FORTH et al.: "Allgemeine und Spezielle Pharmakologie und Toxikologie", edition 5, 1987, pages 680-691: "Antimykotika", BI Wissenschaftsverlag, Mannheim, DE, see the whole document	11-27
P,X	US,A,5032404 (LOPEZ-BERESTEIN et al.) 16 July 1991, see the whole document	28-36
X	EP,A,0260811 (VESTAR, INC.) 23 March 1988, see abstract; claims	28-36
X	WO,A,8806440 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 7 September 1988, see page 11, line 28 - page 13, line 25; page 14, line 24 - page 15, line 19; claims	28-36
X	EP,A,0317120 (VESTAR, INC) 24 May 1989, see the whole document	28-36

FURTHER INFORMAT CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers 1-27, 37-48 because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-27,37-48 are directed to a method of treatment of (diagnostic method practised on) the human/ animal body (PCT Rule 39.1(iv)) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claim numbers because they relate to parts of the International application that do not comp with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9200057 SA 55907

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/04/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A- 5032404	16-07-91	None		
EP-A- 0260811	23-03-88	AU-B-	606880	21-02-91
		AU-A-	7716087	25-02-88
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		JP-A-	63066123	24-03-88
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		AU-A-	1491788	26-09-88
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		JP-T-	2502459	09-08-90
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		DE-A-	3864495	02-10-91
		JP-A-	1160915	23-06-89